Evolution and spread of *Plasmodium falciparum* mutations associated with resistance to sulfadoxine–pyrimethamine in central Africa: a cross-sectional study

Emilie Guémas, Romain Coppée*, Sandie Ménard*, Milena du Manoir, Sandrine Nsango, Dieudonné Makaba Mvumbi, Emmanuel Nakoune, Carole Else Eboumbou Moukoko, Marielle Karine Bouyou Akotet, Tatfeng Youtchou Mirabeau, Sylvie Manguin, Doudou Malekita Yobi, Jean Akiana, Lady Charlène Kouna, Denise Patricia Mawili Mboumba, Dominique Fatima Voumbo-Matoumona, Alliance-Laure Otam, Pierre-Alain Rubbo, Jean-Pierre Lombart, Elisabeth Kwanai, Olivia Cohen, Xavier Iriart, Lawrence Ayong, Jean Bernard Lekana-Douki, Frédéric Ariey, Antoine Berry*

Summary

Background Efficacy of sulfadoxine–pyrimethamine, the malaria chemoprophylaxis used in pregnant women, and in children when combined with amodiaquine, is threatened by the accumulation of mutations in the *Plasmodium falciparum* dihydropteroate synthase (*pfdhps*) and dihydrofolate reductase (*pfdhfr*) genes. Data on the prevalence of resistant alleles in central Africa and the new *pfdhps* I431V mutation, particularly associated with other mutations to form the *pfdhps* vagKgs allele, are scarce. We explored the frequency and geographical distribution of *pfdhps* and *pfdhfr* mutations in central Africa in 2014–18, and assessed the evolutionary origin of the vagKgs allele.

Methods Samples were collected at 18 health-care centres in seven countries (Angola, Cameroon, Central African Republic, Democratic Republic of the Congo, Gabon, Nigeria, and Republic of the Congo) from patients who showed possible symptoms of malaria between March 1, 2014, and Oct 31, 2018. Samples that were positive for *P falciparum* were transported to a laboratory in Toulouse, France, and genotyped. The frequency of *pfdhfr* and *pfdhps* mutations was studied in 1749 samples. Microsatellites in *pfdhps* flanking regions and whole-genome analysis compared with parasite genomes from the data-sharing network MalariaGEN were performed on samples carrying the vagKgs allele.

Findings Mapping of the prevalence of single nucleotide polymorphisms and corresponding alleles of *pfdhfr* and *pfdhps* showed a substantial spread of alleles associated with sulfadoxine–pyrimethamine resistance in central Africa during the 2014–18 period, especially an increase going west to east in *pfdhps* alleles carrying the K540E and A581G mutations. A high prevalence of the *pfdhps* I431V mutation was observed in Cameroon (exceeding 50% in the northern region) and Nigeria. Genomic analysis showed a recent African emergence and a clonal expansion of the most frequent *pfdhps* vagKgs allele.

Interpretation Reduced sulfadoxine–pyrimethamine efficacy due to increased resistance is a worrying situation, especially because the malaria transmission level is high in central Africa. Although the resistance phenotype remains to be confirmed, the emergence and spread of the vagKgs allele in west and central Africa could challenge the use of sulfadoxine–pyrimethamine.

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*Contributed equally

Institut Toulousain des Maladies Infectieuses et Inflammatoires (Infinity), Université de Toulouse, CNRS UMR5051, INSERM UMR 1291, UPS, Toulouse, France (E Guémas PharmD, S Ménard PhD, M du Manoir PhD, A-L Otam MSc, O Cohen MD, X Iriart PhD, Prof A Berry PhD); **Département de Parasitologie et Mycologie, CHU Toulouse, Toulouse, France** (E Guémas, X Iriart, A Berry); **LAAS-CNRS, Université de Toulouse, CNRS, UPS, Toulouse, France** (E Guémas); **Université Paris Cité and Sorbonne Paris Nord, INSERM, IAME, Paris, France** (R Coppée PhD); **Faculté de Médecine et des Sciences Pharmaceutiques, Université de Douala, Douala, Cameroon** (S Nsango PhD, Prof C E Eboumbou Moukoko PhD); **Malaria Research Unit, Centre Pasteur du Cameroun, Yaoundé, Cameroon** (S Nsango, Prof C E Eboumbou Moukoko, L Ayong PhD); **Department of Basic Sciences, Faculty of Medicine, University of Kinshasa, Kinshasa, Democratic Republic of the Congo** (D Makaba Mvumbi PhD, D Malekita Yobi PhD); **Institute for Medical Immunology, Université Libre de Bruxelles, Brussells, Belgium** (D Makaba Mvumbi); **Institut Pasteur de Bangui, Bangui, Central African Republic** (E Nakoune PhD, P-A Rubbo PhD, J-P Lombart PharmD); **Département de Parasitologie Mycologie Médecine Tropicale, Faculté de Médecine de l´Université des Sciences de la**

Introduction

Pregnancy-associated malaria is a major cause of maternal anaemia, prematurity, and low birthweight, which can lead to maternal or neonatal death.[1] The antimalarial sulfadoxine–pyrimethamine combination is used throughout Africa for intermittent preventive treatment in pregnancy and is associated with substantial reductions in malaria burden. Moreover, seasonal malaria chemoprevention with sulfadoxine–pyrimethamine plus amodiaquine has been implemented in 13 countries in the Sahel subregion, representing 45 million children younger than 5 years who were treated in 2021.[2] This policy has rapidly led to a reduction in malaria hospitalisations and deaths.[3]

However, *Plasmodium falciparum* has developed resistance to sulfadoxine–pyrimethamine due to point mutations in the dihydropteroate synthase (*pfdhps*) and dihydrofolate reductase (*pfdhfr*) genes encoding enzymes involved in the obligate folate biosynthesis pathway and targeted by sulfadoxine and pyrimethamine, respectively.[4] The *pfdhfr* C50, N51I, C59R, S108N, I164 triple mutant (CirnI allele) confers a high level of resistance to pyrimethamine and spread in Africa in the 1980s from a single ancestor originating in southeast Asia.[5] Similarly

Santé, Libreville, Gabon (Prof M K Bouyou Akotet PhD, D P Mawili Mboumba PhD); Centre de Recherche Biomédicale en Pathogènes Infectieux et Pathologies Associées, CREIPA, Université des Sciences de la Santé, Libreville, Gabon (M K Bouyou Akotet, D P Mawili Mboumba); Department of Medical Laboratory Science, Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Wilberforce Island, Nigeria (Prof T Y Mirabeau PhD); Hydro Sciences Montpellier, Université de Montpellier, CNRS, IRD, Montpellier, France (Prof S Manguin PhD); Laboratoire National de Santé Publique, Université Marien Ngouabi, Brazzaville, Republic of the Congo (J Akiana PhD, D F Voumbo-Matoumona PhD); Unité d'Evolution Epidémiologie et Résistances Parasitaires, Centre Interdisciplinaire de Recherches Médicales de Franceville, Franceville, Gabon (L C Kouna PhD, D F Voumbo-Matoumona, Prof J B Lekana-Douki PhD); Département de Parasitologie-Mycologie, Université des Sciences de la Santé, Libreville, Gabon (L C Kouna, D F Voumbo-Matoumona, Prof J B Lekana-Douki); Coordination diocésaine de la Santé, Diocèse de Maroua-Mokolo, Maroua, Cameroon (E Kwanai BSc); INSERM U1016, Institut Cochin, Laboratoire de Parasitologie-Mycologie, Hôpital Cochin, AP-HP, Université Paris Cité, Paris, France (Prof F Ariey PhD)

Correspondence to: Prof Antoine Berry, Institut Toulousain des Maladies Infectieuses et Inflammatoires (Infinity), Université de Toulouse, CNRS UMR5051, INSERM UMR 1291, UPS, Toulouse 31300, France
berry.a@chu-toulouse.fr

Research in context

Evidence before this study

The antimalarial sulfadoxine–pyrimethamine combination is used throughout Africa for intermittent preventive treatment in pregnancy and is associated with substantial reductions in malaria burden. In combination with amodiaquine, sulfadoxine–pyrimethamine is also used for seasonal malaria chemoprevention in children younger than 5 years. However, over time, *Plasmodium falciparum* has developed resistance to sulfadoxine–pyrimethamine due to point mutations in the dihydropteroate synthase (*pfdhps*) and dihydrofolate reductase (*pfdhfr*) genes. The accumulation of mutations in these genes is positively correlated with the level of resistance to sulfadoxine–pyrimethamine. So far, two *pfdhps* resistance genotypes are of concern due to their actual or potential impact on the efficacy of malarial prophylaxis. First, the *pfdhps* allele harbouring both A437G and K540E mutations, either combined or not with A581G, diminishes the efficacy of sulfadoxine–pyrimethamine prophylaxis. These genotypes, which are prevalent in eastern and southern Africa, could potentially spread by contiguity to central Africa. We searched PubMed on June 15, 2023, for articles published since Jan 1, 2010, using the search terms "((malaria) AND (pfdhps OR dhps)) AND (K540E or 540E or A581G or 581G) AND (Cameroon OR Congo OR Democratic Republic of the Congo OR Gabon OR Central African Republic OR Angola)". Of the 28 publications found, 18 corresponded to prevalence surveys from identified sites but not from samples collected after therapeutic pressure by sulfadoxine–pyrimethamine (six in the Democratic Republic of the Congo, six in Cameroon, five in Angola, one in Gabon). None of these articles were able to provide an exhaustive view of the prevalence of *pfdhps* resistance alleles in this very vast region. Second, a novel *pfdhps* I431V mutation was described in 2009 in some regions of central and western Africa. This mutation was mostly associated with four other substitutions (S436A, A437G, A581G, and A613S), forming the *pfdhps* vagKgs quintuple mutant. We searched PubMed on June 15, 2023, for articles published since database inception, using the search terms "((malaria) AND (pfdhps or dhps)) AND (I431V or 431V)". 17 studies reported the *pfdhps* I431V mutation but none of them provided information about its origin and reason for emergence or its impact on clinical resistance to sulfadoxine–pyrimethamine. The lack of data prompted us to conduct this study.

Added value of this study

We mapped the prevalence of single nucleotide polymorphisms and corresponding alleles of the *pfdhfr* and *pfdhps* genes from 1749 samples collected in a wide-ranging area of sub-Saharan Africa (18 sites across six countries in central Africa and in one neighbouring country, Nigeria) over the 2014–18 period. The prevalence of the *pfdhps* K540E mutation, with or without the additional *pfdhps* A581G mutation, has considerably increased since previous surveys with an east to west gradient. We also describe the distribution of the I431V mutation, which is highly prevalent in Nigeria and Cameroon, particularly in northern Cameroon (>50%). By compiling data from this study with information from the data-sharing network MalariaGEN and many other studies, a total of 12 270 *pfdhps* sequences from 36 countries were obtained, providing a comprehensive assessment of the prevalence and evolutionary origin of the *pfdhps* I431V mutation. The *pfdhps* I431V mutation has spread across 15 countries in west and central Africa but is not found in east Africa and other malaria-endemic continents. We show the unique emergence of this mutation in west or central Africa and the non-association of the vagKgs allele with specific mutations or gene amplification elsewhere in the genome. None of the samples contained both *pfdhps* I431V and K540E mutations. The opposite mapping of K540E and I431V mutations could suggest that I431V plays a similar role in west and central Africa to K540E in east Africa—namely, a marker of high resistance to sulfadoxine–pyrimethamine.

Implications of all the available evidence

The increased prevalence of alleles carrying the K540E mutation, A581G mutation, or both reduces the efficacy of sulfadoxine–pyrimethamine-based prophylaxis in central Africa, one of the most malaria-affected areas in Africa. Furthermore, the recent and widespread emergence of the I431V mutation and its predominant vagKgs allele is a worrisome reality. Indeed, the absence of vagKgs-associated molecular events, as reported in our study, suggests that the selection of parasites carrying the vagKgs allele is probably only due to its phenotypic advantage. Therefore, there is an urgent need for clinical data to confirm that the vagKgs allele confers an increased level of resistance to sulfadoxine–pyrimethamine. The resistant phenotype of the vagKgs allele, if confirmed, would require reconsideration of sulfadoxine–pyrimethamine chemoprophylaxis.

in *pfdhps*, the A437G mutation alone or in combination with K540E is responsible for sulfadoxine–pyrimethamine treatment failures.[4] Geographical distribution is heterogeneous across Africa with, historically, a west and central Africa pattern for the single A437G mutation but an eastern and southern Africa pattern for the A437G and K540E combination.[4] These different alleles emerged independently in the 1990s and, unlike the *pfdhfr* alleles, originated from Africa.[6]

The *pfdhps* I431V mutation was first described at low frequency in Nigeria in 2007,[7] then in Cameroon in 2010,[8] and later in several central and west African countries.[9] This mutation was mostly found in a *pfdhfr*/*pfdhps* CirnI/vagKgs octuple mutant (N51I, C59R, and S108N in *pfdhfr* and I431V, S436A, A437G, A581G, and A613S in *pfdhps*). To date, little is known about this I431V mutation and its associated alleles, both in terms of geographical distribution and

prevalence as well as its effect on clinical sulfadoxine–pyrimethamine resistance.

In our study, we explored the frequency and geographical distribution of *pfdhps* and *pfdhfr* mutations in central Africa during 2014–18. We also assessed the evolutionary origin of selected parasites harbouring the vagKgs allele using whole-genome sequencing.

Methods

Study design and sample collection

This study was carried out between March 1, 2014, and Oct 31, 2018, at 18 sites in six central African countries (Angola, Cameroon, Central African Republic, Democratic Republic of the Congo, Gabon, and Republic of the Congo) and one neighbouring country (Nigeria; appendix p 20). The study was approved by the ethics committee in each country and by the French National INSERM Ethics Committee.

Patients consulting in the health-care centres taking part in the study who had symptoms suggestive of malaria were tested for malaria (thick smear or immuno-chromatographic rapid diagnostic test). Patients with a confirmed *P falciparum*-positive test were enrolled as volunteers after giving their informed consent. Patients with severe malaria according to WHO criteria[10] were excluded from the study. No parasite density criteria were requested because only successfully genotyped samples were analysed. A drop of previously collected blood was placed on filter paper (Whatman FTA Elute Card; Whatman, Middlesex, UK), stored dry, and transferred to the Infinity laboratory in Toulouse (France). DNA was extracted according to the manufacturer's instructions and stored at –20°C until use.

Single nucleotide polymorphism (SNP) genotyping

PCR amplification and Sanger sequencing of *pfdhfr* and *pfdhps* genes were performed as previously described.[8] For samples with mixed infections, we analysed SNPs and alleles only if one allele was present with a 2:1 ratio (ie, the signal of the minority sequence was less than half the intensity of the majority one) and considered as the majority allele. The mutated codons are written in lower case. 95% CIs of proportions were calculated using the Wilson score method without continuity correction.

PCR microsatellite analysis

The evolutionary origins of *pfdhps* alleles were investigated by analysing the polymorphism of three microsatellites in the flanking region located at 0·8 kb, 4·3 kb, and 7·7 kb from the 3′ end of *pfdhps*. We selected samples containing the alleles of interest and a representative sampling of the other alleles. Semi-nested PCRs were performed independently for each micro-satellite, as previously described.[11] PCR fragment size was determined by electrophoresis on an ABI 3730 XL DNA Analyser (Applied Biosystems, Waltham, MA, USA) and analysed with Peak Scanner version 1.0 (Thermo Fisher Scientific, Waltham, MA, USA). In mixed samples, the data were considered missing at this locus for this sample.

Genetic diversity using microsatellites was analysed by calculating the expected heterozygosity (*He*) with FSTAT version 2.9.4.[12] Microsatellite haplotypes of the two most closely linked markers were ranked, first to allele size at locus 0·8 kb and then by allele size at locus 4·3 kb. A high diversity of microsatellite haplotypes suggested an absence of allele selection, whereas a loss in microsatellite haplotype diversity was evidence of positive selection.[6]

Whole-genome sequencing

See Online for appendix

The selective whole-genome amplification approach was used to enrich *P falciparum* DNA from total DNA extracts before whole-genome sequencing, as previously described.[13] Amplified products were cleaned using Agencourt Ampure XP beads (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions.

After mechanically shearing 250 ng of total DNA with the Covaris S220 Focused Ultrasonicator (Covaris, Woburn, MA, USA), genomic DNA libraries were constructed for high-throughput sequencing using the KAPA HyperPrep Library Preparation Kit (Kapa Biosystems, Woburn, MA, USA) then checked for quantity and quality using Qubit (Invitrogen, Waltham, MA, USA) for concentration and BioAnalyser 2100 (Agilent, Santa Clara, CA, USA) for fragment size. Libraries were sequenced as paired-end 150-bp reads using an Illumina NextSeq 500 (San Diego, CA, USA) instrument at the GENOM'IC facility at the Cochin Institute (Paris, France).

Whole-genome analyses

Raw sequence data were first subjected to standard Illumina quality control procedures. Raw reads were then mapped to the *P falciparum* 3D7 reference genome v39 using the Burrows-Wheeler Aligner software version 0.7.15.[14] An analysis pipeline for variant discovery and genotyping, including stringent quality control filters that took into account the unusual features of the *P falciparum* genome, was developed similarly to that proposed by the MalariaGEN consortium (appendix p 2).[15]

The genetic variation of our samples containing the alleles of interest was compared to that of previously sequenced *P falciparum* isolates.[15] A discriminant analysis of principal components was performed on samples originating from Africa, South America, and southeast Asia using the adegenet package in R.[16] For principal component analysis, we subsequently focused on African countries harbouring at least one vagKgs-carrying isolate in the MalariaGEN database. The analysis was restricted on SNPs genotyped in a range of 50 kb upstream or downstream of *pfdhps* using BEDtools v.2.26.0.[17] An SNP

	Number of *pfdhps* sequences	Prevalence of *pfdhps* allele, % (95% CI), n											
		ISAKAA	IaAKAA	ISgKAA	IagKAA	IagKAs	ISgeAA	ISgegA	ISAegA	vagKAA	vagKAs	vagKgs	Other alleles*
Angola													
Balombo (2017)	58	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	56·9% (44·1–68·8); 33	19·0% (10·9–30·9); 11	0·0% (0·0–6·2); 0	24·1% (15·0–36·5); 14	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0
Cameroon													
Douala (2014)	86	1·2% (0·2–6·3); 1	10·5% (5·6–18·7); 9	65·1% (54·6–74·3); 56	5·8% (2·5–12·9); 5	0·0% (0·0–4·3); 0	0·0% (0·0–4·3); 0	0·0% (0·0–4·3); 0	0·0% (0·0–4·3); 0	2·3% (0·6–8·1); 2	0·0% (0·0–4·3); 0	7·0% (3·2–14·4); 6	8·2% (4·0–15·9); 7
Garoua (2014)	104	10·6% (6·0–18·0); 11	33·7% (25·3–43·2); 35	26·9% (19·3–36·2); 28	5·8% (2·7–12·0); 6	3·8% (1·5–9·5); 4	1·0% (0·2–5·2); 1	0·0% (0·0–3·6); 0	0·0% (0·0–3·6); 0	1·9% (0·5–6·7); 2	1·9% (0·5–6·7); 2	9·6% (5·3–16·8); 10	4·8% (2·1–10·8); 5
Maroua (2017)	140	5·0% (2·4–10·0); 7	15·0% (10·0–21·8); 21	20·7% (14·8–28·2); 29	0·7% (0·1–3·9); 1	2·9% (1·1–7·1); 4	0·0% (0·0–2·7); 0	0·0% (0·0–2·7); 0	0·0% (0·0–2·7); 0	5·0% (2·4–10·0); 7	0·7% (0·1–3·9); 1	48·6% (40·4–56·8); 68	1·4% (0·4–5·1); 2
Tibati (2015)	99	2·0% (0·6–7·1); 2	33·3% (24·8–43·1); 33	39·4% (30·3–49·2); 39	12·1% (7·1–20·0); 12	0·0% (0·0–3·7); 0	0·0% (0·0–3·7); 0	0·0% (0·0–3·7); 0	0·0% (0·0–3·7); 0	6·1% (2·8–12·6); 6	1·0% (0·2–5·5); 1	4·0% (1·6–9·9); 4	2·0% (0·6–7·1); 2
Yaoundé (2015)	77	2·6% (0·7–9·0); 2	14·3% (8·2–23·8); 11	55·8% (44·7–66·4); 43	13·0% (7·2–22·3); 10	2·6% (0·7–9·0); 2	0·0% (0·0–4·8); 0	0·0% (0·0–4·8); 0	0·0% (0·0–4·8); 0	5·2% (2·0–12·6); 4	0·0% (0·0–4·8); 0	6·5% (2·8–14·3); 5	0·0% (0·0–4·8); 0
Central African Republic													
Bangui (2018)	157	1·9% (0·7–5·5); 3	48·4% (40·7–56·2); 76	28·7% (22·2–36·2); 45	9·6% (5·9–15·2); 15	0·6% (0·1–3·5); 1	7·6% (4·4–12·9); 12	1·9% (0·7–5·5); 3	0·0% (0·0–2·4); 0	0·0% (0·0–2·4); 0	0·0% (0·0–2·4); 0	1·3% (0·4–4·5); 2	0·0% (0·0–2·4); 0
Democratic Republic of the Congo													
Kabare (2018)	74	2·7% (0·7–9·3); 2	2·7% (0·7–9·3); 2	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0	40·5% (30·1–51·9); 30	54·1% (42·8–64·9); 40	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0	0·0% (0·0–4·9); 0
Karawa (2018)	58	1·7% (0·3–9·1); 1	51·7% (39·2–64·1); 30	24·1% (15·0–36·5); 14	3·4% (1·0–11·7); 2	0·0% (0·0–6·2); 0	5·2% (1·8–14·1); 3	13·8% (7·2–24·9); 8	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0
Kinshasa (2018)	84	6·0% (2·6–13·2); 5	1·2% (0·2–6·4); 1	65·5% (54·8–74·8); 55	2·4% (0·7–8·3); 2	0·0% (0·0–4·4); 0	16·7% (10·2–26·1); 14	6·0% (2·6–13·2); 5	0·0% (0·0–4·4); 0	0·0% (0·0–4·4); 0	0·0% (0·0–4·4); 0	2·4% (0·7–8·3); 2	0·0% (0·0–4·4); 0
Kisangani (2018)	81	1·2% (0·2–6·7); 1	7·4% (3·4–15·2); 6	30·9% (21·9–41·6); 25	2·5% (0·7–8·6); 2	0·0% (0·0–4·5); 0	32·1% (22·9–42·9); 26	24·7% (16·6–35·1); 20	1·2% (0·2–6·7); 1	0·0% (0·0–4·5); 0	0·0% (0·0–4·5); 0	0·0% (0·0–4·5); 0	0·0% (0·0–4·5); 0
Mbandaka (2018)	59	0·0% (0·0–6·1); 0	1·7% (0·3–9·0); 1	57·6% (44·9–69·4); 34	0·0% (0·0–6·1); 0	0·0% (0·0–6·1); 0	8·5% (3·7–18·4); 5	30·5% (20·3–43·1); 18	0·0% (0·0–6·1); 0	0·0% (0·0–6·1); 0	0·0% (0·0–6·1); 0	0·0% (0·0–6·1); 0	1·7% (0·3–9·0); 1
Pangi (2018)	58	1·7% (0·3–9·1); 1	5·2% (1·8–14·1); 3	10·3% (4·8–20·8); 6	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	37·9% (26·6–50·8); 22	44·8% (32·7–57·5); 26	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0	0·0% (0·0–6·2); 0
Gabon													
Franceville (2017)	23	0·0% (0·0–14·3); 0	4·3% (0·8–21·0); 1	82·6% (62·9–93·0); 19	8·7% (2·4–26·8); 2	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	4·3% (0·8–21·0); 1	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0
Libreville (2016)	57	0·0% (0·0–6·3); 0	5·3% (1·8–14·4); 3	89·5% (78·9–95·1); 51	1·8% (0·3–9·3); 1	0·0% (0·0–6·3); 0	3·5% (1·0–11·9); 2	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0
Oyem (2017)	84	4·8% (1·9–11·6); 4	7·1% (3·3–14·7); 6	64·3% (53·6–73·7); 54	3·6% (1·2–10·0); 3	0·0% (0·0–4·4); 0	15·5% (9·3–24·7); 13	0·0% (0·0–4·4); 0	0·0% (0·0–4·4); 0	2·4% (0·7–8·3); 2	0·0% (0·0–4·4); 0	2·4% (0·7–8·3); 2	0·0% (0·0–4·4); 0
Nigeria													
Port Harcourt (2016)	57	3·5% (1·0–11·9); 2	7·0% (2·8–16·7); 4	45·6% (33·4–58·4); 26	8·8% (3·8–18·9); 5	3·5% (1·0–11·9); 2	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	0·0% (0·0–6·3); 0	3·5% (1·0–11·9); 2	1·8% (0·3–9·3); 1	22·8% (13·8–35·2); 13	3·5% (1·0–11·9); 2
Republic of the Congo													
Brazzaville (2017)	23	4·3% (0·8–21·0); 1	0·0% (0·0–14·3); 0	69·6% (49·1–84·4); 16	8·7% (2·4–26·8); 2	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	8·7% (2·4–26·8); 2	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	0·0% (0·0–14·3); 0	8·7% (2·4–26·8); 2	0·0% (0·0–14·3); 0

Pfdhps alleles are named according to the amino acid present at positions 431, 436, 437, 540, 581, and 613. The wild-type amino acid is stated in upper case, with the mutated amino acid in lower case. 95% CIs of proportions were calculated using the Wilson score method without continuity correction. *Other alleles include IfAKAs, IaAKgA, IaAKgs, ISgKgA, ISgKgs, IcAKAA, IhAKAA, ItgKAA, and ISgngA.

***Table:* Prevalence of *pfdhps* alleles produced by Sanger sequencing across 18 sites in seven central African countries**

was considered only when the corresponding position was genotyped in at least 80% of the samples. Population structure was investigated by principal component analysis using the SNPRelate package in R.

From the reads mapped to the 3D7 reference genome, seven microsatellites were genotyped around the *pfdhps* gene on the samples we sequenced and 109 samples from MalariaGEN covering the *pfdhps* alleles ISgKAA, IagKAA, IaAKAA, vagKAA, and vagKgs, as previously described.[18] At least five sequence reads covering a repeated motif and some nucleotides upstream and downstream of the motif were required to unambiguously interpret the length of the microsatellite.

Per-gene copy number was assessed for 76 *pfdhps* vagKgs-carrying samples in 5427 genes using PlasmoCNVScan.[19]

The within-sample *F* statistic (*Fws*) was applied to determine whether each sample was monoclonal or polyclonal. The *Fws* was calculated using the moimix (version 0.0.2.9001) R package[20] and samples with *Fws* of less than 0·95 were considered polyclonal.

Genome-wide association study (GWAS)

A GWAS was performed using a script developed by Biogenosis, as previously described.[21] Fisher's exact test was used to identify significant SNP differences between parasite lines carrying or not carrying the *pfdhps* vagKgs allele; and between the different vagKgs sublineages (evidenced by principal component analysis).

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We evaluated the prevalence of *pfdhfr* and *pfdhps* alleles across 18 sites in seven countries: Angola, Cameroon, Central African Republic, Democratic Republic of the Congo, Gabon, Nigeria, and Republic of the Congo (appendix p 20). Of 1749 samples from patients with malaria at these sites, 1477 sequences were successfully obtained for *pfdhfr* and 1379 for *pfdhps*. Successful genotyping for both genes was determined for 1194 samples.

Six *pfdhfr* alleles were identified, combining mutations at codons 51, 59, 108, and 164 (appendix pp 3–4). The *pfdhfr* CirnI triple mutant allele had a frequency ranging from 80·6% to 100% across all the study sites except in Angola (Balombo; 23·7%). 20 *pfdhps* alleles were observed, combining mutations at codons 431, 436, 437, 540, 581, and 613 (table; appendix p 5). The A437G single mutant (ISgKAA) was the major *pfdhps* allele at more than half of the sites, with a frequency between 39·4% and 89·5%. The K540E mutation was mainly observed in the eastern and southern sites of our study (Democratic Republic of the Congo, Angola, and Oyem in Gabon) with a prevalence ranging from 15·5% to 94·6%.

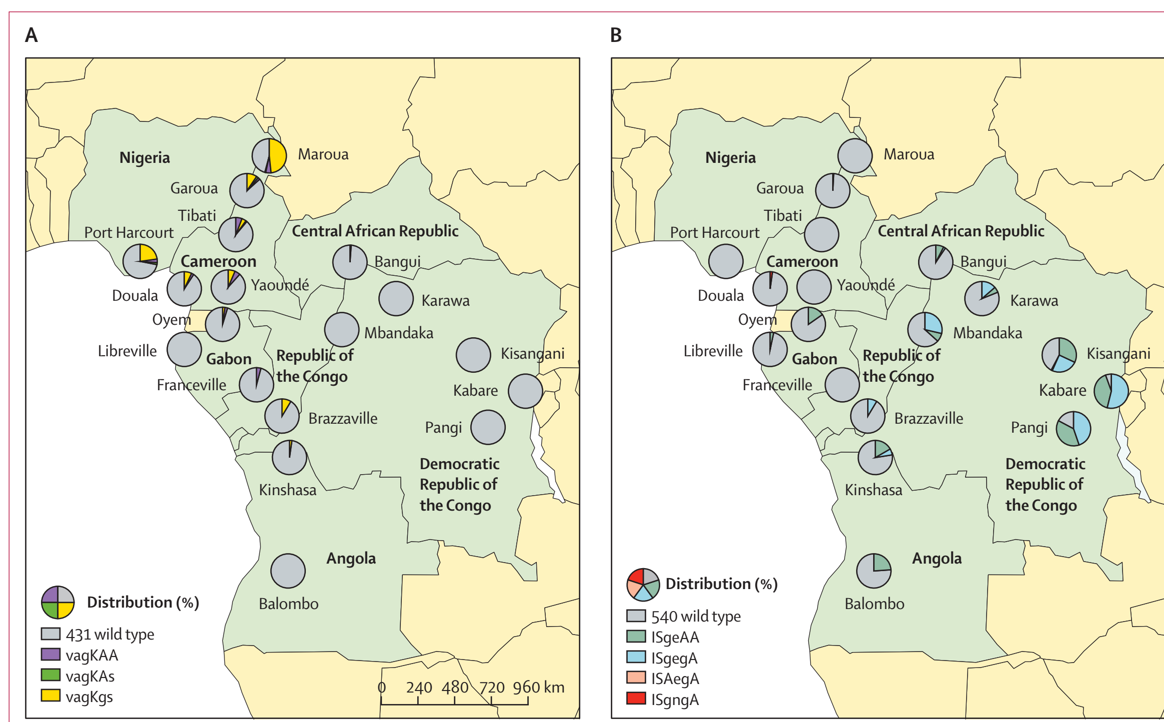

Figure 1: **Distribution of *pfdhps* alleles containing I431V (A) or K540E/N (B) mutations in the *pfdhps* gene**
Pfdhps alleles are named according to the amino acid present at positions 431, 436, 437, 540, 581, and 613.

The *pfdhps* I431V mutation was heterogeneously distributed among study sites, mostly found in the northwestern sites of our study and especially in Cameroon (from 9·3% in Douala to 54·3% in Maroua) and Nigeria (28·1% in Port Harcourt). By contrast, the frequency of I431V was lower in Republic of the Congo (8·7%), Gabon (up to 4·8% in Oyem), Democratic Republic of the Congo (up to 2·4% in Kinshasa), and Central African Republic (1·3%), with none observed in Angola. Of note, the I431V mutation had a geographical distribution that was the opposite of the K540E mutation and was never observed in combination together (figure 1; appendix p 5). The vagKgs quintuple mutant was the most common allele carrying the I431V mutation with a frequency of 78·6%, whereas the two other alleles, vagKAA and vagKAs, represented only 18·0% and 3·4%, respectively. The vagKgs quintuple mutant was observed in combination with the *pfdhfr* CirnI triple mutant allele in 93·6% of samples (appendix p 6). The vagKgs quintuple mutant was mainly found in Maroua (Cameroon) and Port Harcourt (Nigeria), representing 48·6% and 22·8% of the *pfdhps* alleles, respectively (table).

After compiling data from our study, MalariaGEN (appendix pp 7–8), and several other studies (appendix pp 9–10), 12 270 *pfdhps* sequences from 36 countries in Africa, South America, and southeast Asia were obtained. The I431V mutation was found in only 617 samples from 15 countries in central and west Africa (figure 2). The frequency of the mutation was found to be the highest in Cameroon, Nigeria, Chad, and Benin. 12 alleles carrying the I431V mutation were identified. As in our field study, the three major alleles were vagKgs (identified in 422 [68·4%] of samples), vagKAA (78 [16·9%]), and vagKAs (23 [4·5%]).

To better understand the selection process, we characterised microsatellite polymorphisms flanking the *pfdhps* gene at the 0·8 kb locus for 722 samples from this study, at 4·3 kb for 781 samples, and at 7·7 kb for 719 samples. By combining the two most closely related markers (0·8 kb and 4·3 kb),[6] we obtained 53 microsatellite haplotypes across 687 samples (appendix p 11). The *pfdhps* wild-type ISAKAA allele and the S436A single (IaAKAA) mutant had an expected *He* for the 0·8 kb locus close to 1 (0·937 and 0·873, respectively), with 15 and 25 different microsatellite haplotypes, respectively (figure 3; appendix p 12). In contrast with these two alleles, genetic diversity was gradually lowered with the accumulation of *pfdhps* mutations. *He* for the 0·8 kb locus decreased to 0·625 for the A437G single mutant and to 0·515 and 0·402 for the *pfdhps* K540E-associated alleles, ISgeAA and ISgegA, respectively. The loss of diversity was considerably more pronounced for the *pfdhps* alleles associated with the I431V mutation, with *He* values at the 0·8 kb locus ranging from 0·177 (vagKAA) to 0·060 (vagKgs), suggestive of a recent selection. Of note, the vagKAA and vagKgs mutants did not share the same microsatellite haplotype, suggesting a different I431V-carrying ancestral mutant lineage depending on the

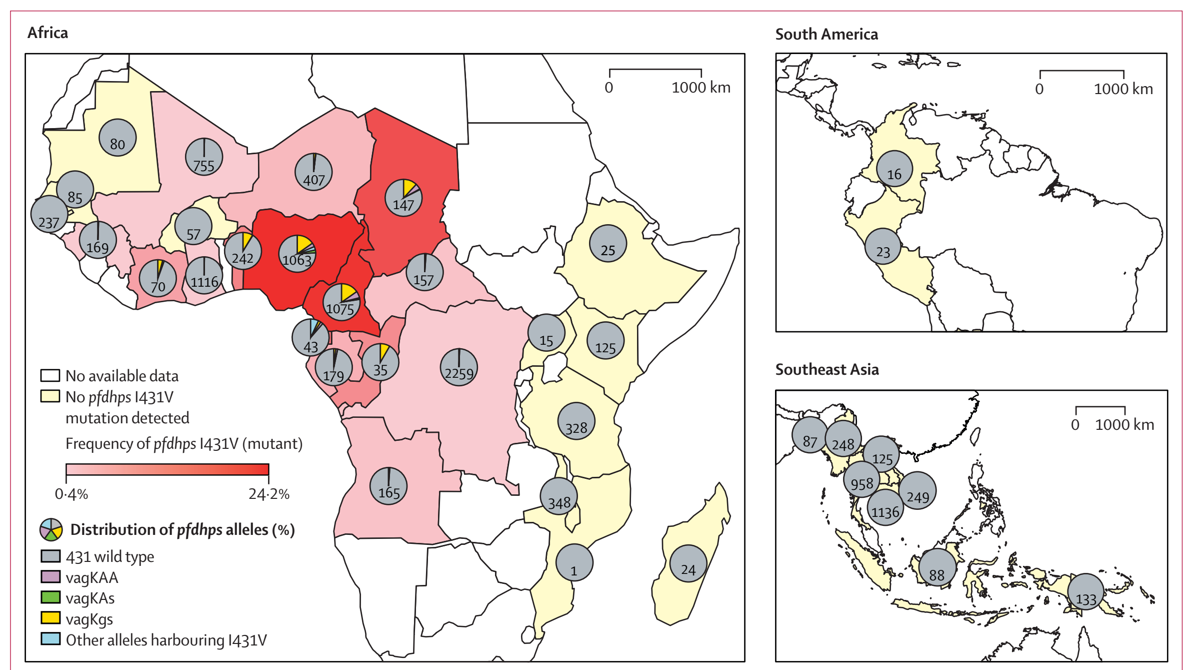

Figure 2: **Global distribution of the *pfdhps* I431V mutation**
12 270 samples were included, including 1379 from this study (2014–18 period), 6780 from MalariaGEN (2002–15 period), and 4111 from several other studies (appendix pp 9–10). *Pfdhps* alleles are named according to the amino acid present at positions 431, 436, 437, 540, 581, and 613. The total numbers of samples from each country are shown on the pie charts.

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allele, whereas the vagKAs allele shared microsatellite haplotype 34 (75%) with the vagKAA mutant but also microsatellite haplotype 41 (25%) with the vagKgs mutant.

To elucidate the geographical emergence of the I431V mutation, whole genomes of 18 *pfdhps* I431V isolates (including 16 with the vagKgs allele) from this study were sequenced and their genetic diversity was compared to 260 samples retrieved from MalariaGEN collections encompassing 60 samples from Asia, ten from Oceania, 20 from South America, and 170 from Africa (appendix pp 13–14). Using 17 380 high-quality SNPs located in a range of 100 kb upstream and downstream of *pfdhps*, the discriminant analysis of principal components highlighted distinct genetic backgrounds between Africa, Asia, South America, and Oceania. All the samples carrying the *pfdhps* I431V mutation clustered with all other African samples (figure 4A), confirming a local emergence of the I431V mutation in Africa.

To further explore the genetic diversity of vagKgs mutants, a GWAS based on the presence (n=92) or absence (n=484) of the vagKgs allele and covering a total of 2 217 669 SNPs across the 14 *P falciparum* chromosomes identified only one locus showing signs of selection (figure 4B). This region was located between positions 500 000 and 550 000 of chromosome 8 and included the *pfdhps* gene and its 5′ region. Similarly, the analysis of the extended haplotype homozygosity around the *pfdhps* gene and centred on the I431V mutation showed that parasites carrying this mutation evolve under positive selection (appendix p 21). The principal component analysis of 12 667 SNPs located in a range of 50 kb upstream or downstream of *pfdhps* revealed two distinct vagKgs populations (figure 4C): Group 1 (GP1) mainly represented by the samples we sequenced and GP2 almost exclusively consisting of parasites from MalariaGEN data. The vagKgs samples in the core of the distribution were mostly polyclonal, explaining why they did not cluster with either GP1 or GP2 (appendix pp 15–17). The GP1 and GP2 patterns were not explained by the geographical origin (country or site) of the samples, the time of collection, or the multiplicity of infection (appendix p 22). Moreover, performing principal component analyses on SNPs located on other chromosomes of *P falciparum* or in another region of chromosome 8 distant from *pfdhps* failed to distinguish the vagKgs (either GP1 or GP2) from the other samples (appendix p 23), consistent with the aforementioned GWAS. To better understand the genetic difference between GP1 and GP2, we explored seven microsatellites around the *pfdhps* gene (appendix p 24). Four microsatellites were fully identical (the three loci located downstream of the *pfdhps* gene and the closest locus upstream of the *pfdhps* gene), whereas the other three microsatellites, located furthest upstream of *pfdhps*, were different. Additionally, we performed a GWAS between GP1 and GP2 (using only monoclonal samples) and observed that more than 75% of differential SNPs were located in the 50 kb upstream of *pfdhps* (appendix p 25). Such a result suggests that GP1 and GP2 parasites shared the same genetic background, consistent with a single emergence of the vagKgs allele, whereas the strong difference upstream of *pfdhps* could be the result of a recombination event after the vagKgs allele selection process.

To investigate whether vagKgs-carrying isolates harboured compensatory or selection-enhancing mutations, we studied polymorphism in the 11 genes present in the 50-kb region at the 5′ end of *pfdhps* identified by GWAS (figure 4D). When focusing only on non-synonymous mutations from the GWAS, in addition to SNPs in the *pfdhps*, three codons were notable: SNP Q169H in the ATP-dependent RNA helicase DBP1, SNP E789G in the JmjC domain-containing protein 1, and SNP K68N in a conserved *Plasmodium* protein of unknown function which is always found as wild type (K68) in the vagKgs group (appendix pp 18–19). These three genes are not known to be involved in metabolic pathways that might lead to sulfadoxine–pyrimethamine resistance. Furthermore, we did not detect any amplified gene specific to vagKgs-carrying samples (Figure 4E)

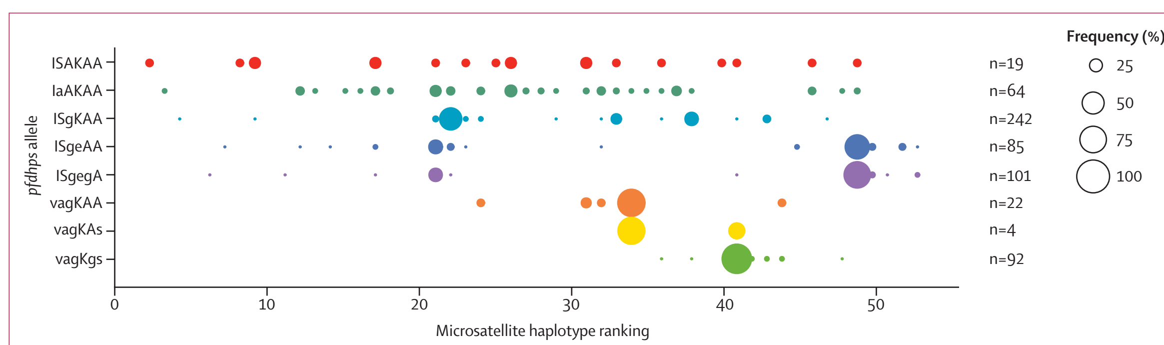


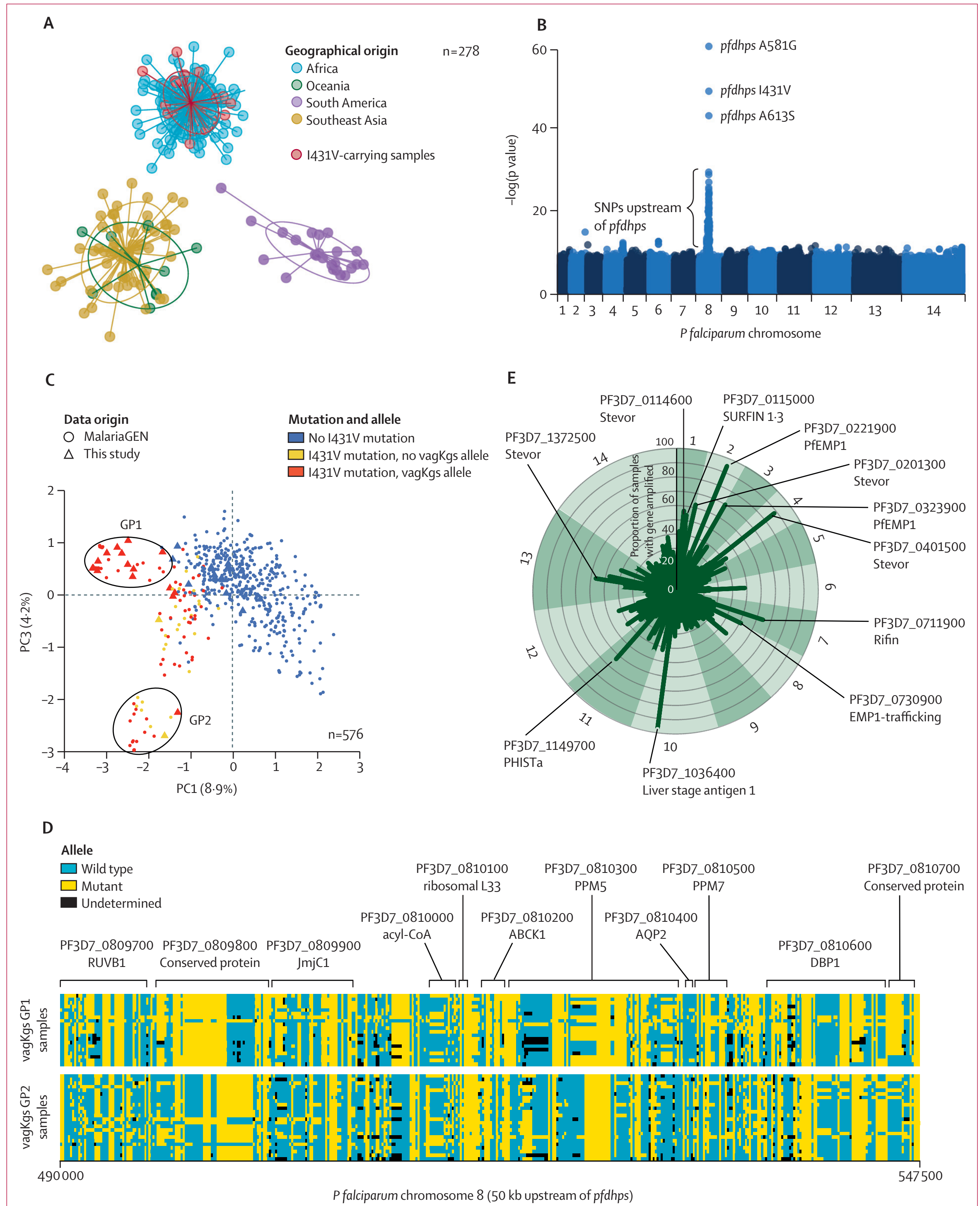
Figure 3: **Frequency of the microsatellite haplotypes associated with wild-type *pfdhps* and some mutant alleles**
Microsatellite haplotypes have been ranked first according to allele size at locus 0·8 kb, then by allele size at locus 4·3 kb and listed along the x-axis. *Pfdhps* alleles are named according to the amino acid present at positions 431, 436, 437, 540, 581, and 613. The wild-type amino acid is stated in upper case, with the mutated amino acid in lower case.

except the liver stage antigen 1 (PF3D7_1036400), which is commonly amplified in isolates.[22]

Our conclusion is that the 50-kb location of the 5′ end of the vagKgs allele is associated with a hitchhiking effect. The appearance of the *pfdhps* vagKgs allele does not depend on any specific genetic background, stabilising compensatory mutation, or gene amplification.

Discussion

With a steady increase in resistance to sulfadoxine–pyrimethamine compromising the effectiveness of



intermittent preventive treatment in pregnancy and seasonal malaria chemoprevention, regular surveys on the efficacy of these policies and monitoring of molecular markers of resistance are essential. The first part of this study explored the geographical distribution of *pfdhps* and *pfdhfr* mutations by collecting more than 1500 samples across most central African countries, an area for which there are much fewer data than in western, eastern, or southern Africa. Previous studies[4] and our results show that the prevalence of the *pfdhfr* CirnI allele is now fixed at a very high level (>95%) in southeastern Nigeria, southern Cameroon, and Gabon. Elsewhere, the increased frequency of this allele was observed in a concentric progression with values ranging from 80% to 90%, except in Balombo, Angola (23·7%).

In southeastern Nigeria, southern Cameroon, and Gabon, we also observed increased prevalence of the *pfdhps* A437G mutation at 80–95%, alone or combined with S436A, forming the ISgKAA or IagKAA alleles. These *pfdhfr/pfdhps* haplotypes, CirnI/ISgKAA or CirnI/IagKAA, confer the first level of resistance to sulfadoxine–pyrimethamine[23] but do not impact the efficacy of intermittent preventive treatment in pregnancy in terms of low birthweight.[24] However, the impact on maternal anaemia and parasitaemia is not well known. The efficacy of seasonal malaria chemoprevention does not seem to be affected by these genotypes,[9] but such data need to be confirmed. By contrast, the additional *pfdhps* K540E mutation reduces the efficacy of both intermittent preventive treatment of malaria in pregnancy and seasonal malaria chemoprevention,[25,26] and the additional *pfdhps* A581G mutation enhances this resistance.[24,27] Intermittent preventive treatment in pregnancy with sulfadoxine–pyrimethamine is estimated to be no longer effective when the prevalence of K540E is greater than 90% and of A581G is greater than 10%.[24] Our study showed a very high level of resistance in Kabare (Democratic Republic of the Congo; 94·6% for K540E and 54·1% for A581G), consistent with the prevalence previously observed in neighbouring countries (Rwanda, Uganda, and Tanzania).[4] Even if the prevalence of these mutations decreased when moving towards the west, they have considerably increased since previous surveys.[4,28] This trend is likely to dramatically reduce the efficacy of intermittent preventive treatment in pregnancy with sulfadoxine–pyrimethamine in one of the most malaria-affected areas of Africa.

Our survey and analysis of the MalariaGEN dataset and several other studies identified a high frequency of the new *pfdhps* I431V mutation in Cameroon, Nigeria, Chad, and Benin. Although our study did not identify the I431V mutation in Angola (Balombo), the low frequency observed in samples collected in 2019 from Zaire province (2·9%) indicated the continuous spread of this mutation.[29] High prevalence has already been described in Nigeria[30] as well as Chad[9] and this mutation is also found in several west and central African countries, but has never been observed elsewhere in Africa or on other continents. This distribution favours an emergence having occurred in one of these four countries.

Analysis of the evolution of I431V over time is scarce. It is difficult to assess whether the lack of data is due to the absence of the I431V mutation in the field or to an absence of its screening. Furthermore, studies either do not screen for the I431V mutation or are based on non-sequencing methods. Nevertheless, analysis of data retrieved from the literature and from MalariaGEN (based on whole-genome analysis) showed the first sporadic detection of this substitution from 2007 in Nigeria.[7] Moreover, the low level of genetic diversity also supports the idea of a recent selection of the I431V mutation and clonal emergence of the vagKgs allele.

In the absence of compensatory mutations (SNPs or copy number variations) or a related genetic background, we can infer that the selection of the vagKgs allele is solely based on its advantageous phenotype. Given that mutations in the *pfdhfr* and *pfdhps* genes are positively correlated with increasing levels of sulfadoxine–pyrimethamine resistance,[31] we can reasonably expect that this new haplotype would confer a high level of resistance to sulfadoxine–pyrimethamine and has been selected due to extensive use of sulfadoxine–pyrimethamine for intermittent preventive treatment in pregnancy. To date, this hypothesis remains speculative in the absence of clinical studies. However, a study conducted in Cameroon in 2020 showed an over-representation of the CirnI/vagKgs haplotype in the group of pregnant women with acute malaria receiving intermittent preventive treatment in pregnancy with sulfadoxine–pyrimethamine compared with those who

Figure 4: **Genetic analyses of *pfdhps* I431V-carrying samples**
(A) Geographical origin of the I431V mutation based on a discriminant analysis of principal components. The analysis was performed using 17 380 SNPs located in a range of 100 kb upstream and downstream of *pfdhps*, in 260 samples from different continents (MalariaGEN data) and 18 *pfdhps* I431V-carrying mutants from this study. (B) Manhattan plot showing the significance of SNP association in the genome-wide association study. Each point represents one of the 2 217 669 SNPs in a set of 576 samples (92 with the vagKgs allele and 484 without), coloured according to chromosome. The y-axis corresponds to the –log(p value) for the SNP association calculated using Fisher's exact test. (C) Population structure harbouring different *pfdhps* alleles based on a principal component analysis. We focused on African countries with at least one vagKgs-carrying isolate in the MalariaGEN database. 576 samples were included (89 from Benin, 180 from Cameroon, 100 from Ghana, 70 from Côte d'Ivoire, 100 from Mali, and 37 from Nigeria) covering 12 667 SNPs located in a range of 50 kb upstream or downstream of *pfdhps*. 92 samples carried the *pfdhps* vagKgs allele and 113 had the I431V mutation. (D) Comparison of two vagKgs-carrying GP1 and GP2 lineages based on 11 genes upstream of *pfdhps*. Each cell represents a single SNP. Each row corresponds to one isolate. Only monoclonal samples were included for this analysis. (E) Estimation of per-gene copy number for 76 *pfdhps* vagKgs-carrying samples using the PlasmoCNVScan program. Each line represents one of the 5427 genes and corresponds to the percentage of samples with a gene amplification. Numbers around the outside of the circle indicate the *P falciparum* chromosome. The name and identifier of the gene is indicated when the percentage of samples is 50% or more. GP=Group. PC=principal component. *P falciparum*=*Plasmodium falciparum*. SNP=single nucleotide polymorphism.

did not receive prophylaxis, suggesting a high level of resistance to sulfadoxine–pyrimethamine for this mutant.[32] By contrast, Beshir and colleagues[9] conducted a molecular surveillance study of the seasonal malaria chemoprevention policy in the Sahel region and found no evidence of increased prevalence of the vagKgs allele in the target population at 2-year intervals (2016 and 2018) in Chad, Niger, and Nigeria, with an excellent efficacy of the prophylaxis. Unlike mass treatment that relies solely on sulfadoxine–pyrimethamine, this study evaluated the efficacy of seasonal malaria chemoprevention using a combination of sulfadoxine–pyrimethamine and amodiaquine. The use of this drug combination (ie, the presence of amodiaquine) might explain the absence of a selection of parasites that are hyper-resistant to sulfadoxine–pyrimethamine.

Hypothesising that selection of the vagKgs allele is solely related to sulfadoxine–pyrimethamine use, as suggested by the results of our GWAS, is somewhat surprising considering that emergence occurred in Nigeria or Cameroon, in areas known to have had great difficulties for several years (lack of state, poverty, and armed conflicts) that have compromised the implementation of intermittent preventive treatment in pregnancy and seasonal malaria chemoprevention.[33] However, it cannot be ruled out that I431V is a compensatory mutation that would ensure greater performance of the PfDHPS enzyme in the presence of resistance mutations (A437G, A581G, A613S); in this scenario, it could give rise to better fitness of resistant parasites even in the absence of selection pressure by sulfadoxine–pyrimethamine.

Of note, the combination of K540E and I431V mutations has not been observed in our study nor in any published data, suggesting a high fitness cost. The vagKgs haplotype could be to central and western Africa what the ISgegA haplotype is to eastern and southern Africa: a super-resistant parasite, as defined by Naidoo and colleagues.[4] Therefore, further investigations are needed to clarify the extent to which this vagKgs allele contributes to the reduced efficacy of sulfadoxine–pyrimethamine in intermittent preventive treatment in pregnancy or seasonal malaria chemoprevention campaigns. Nevertheless, this study emphasises that the use of sulfadoxine–pyrimethamine selects for mutations, meaning that switching to drug combinations is recommended rather than using sulfadoxine–pyrimethamine alone.

Contributors

RC, SMé, FA, and AB conceptualised and designed the study. SMé and AB wrote the protocols. SN, DMM, EN, CEEM, MKBA, TYM, SMa, JA, EK, and JBL-D organised sample collection. DMY, P-AR, J-PL, LA, LCK, DFV-M, DPMM, and JBL-D performed sample collection. EG, RC, SMé, MdM, A-LO, and FA performed the laboratory experiments. EG, RC, SMé, MdM, OC, XI, FA, and AB analysed the data. EG, RC, SMé, FA, and AB drafted the manuscript. All authors read and approved the final version of the manuscript. RC, SMé, and AB had full access to all the data in the study, and AB had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

The study protocols and dataset will be made available upon reasonable request made to the corresponding author.

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References

1 Desai M, ter Kuile FO, Nosten F, et al. Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis* 2007; **7:** 93–104.
2 WHO. World malaria report 2022. Dec 8, 2022. https://www.who.int/publications/i/item/9789240064898 (accessed Feb 15, 2023).
3 Cairns ME, Sagara I, Zongo I, et al. Evaluation of seasonal malaria chemoprevention in two areas of intense seasonal malaria transmission: secondary analysis of a household-randomised, placebo-controlled trial in Houndé District, Burkina Faso and Bougouni District, Mali. *PLoS Med* 2020; **17:** e1003214.
4 Naidoo I, Roper C. Mapping 'partially resistant', 'fully resistant', and 'super resistant' malaria. *Trends Parasitol* 2013; **29:** 505–15.
5 Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. *Science* 2004; **305:** 1124.
6 Pearce RJ, Pota H, Evehe M-SB, et al. Multiple origins and regional dispersal of resistant *dhps* in African *Plasmodium falciparum* malaria. *PLoS Med* 2009; **6:** e1000055.
7 Sutherland CJ, Fifer H, Pearce RJ, et al. Novel *pfdhps* haplotypes among imported cases of *Plasmodium falciparum* malaria in the United Kingdom. *Antimicrob Agents Chemother* 2009; **53:** 3405–10.
8 Chauvin P, Menard S, Iriart X, et al. Prevalence of *Plasmodium falciparum* parasites resistant to sulfadoxine/pyrimethamine in pregnant women in Yaoundé, Cameroon: emergence of highly resistant *pfdhfr/pfdhps* alleles. *J Antimicrob Chemother* 2015; **70:** 2566–71.
9 Beshir KB, Muwanguzi J, Nader J, et al. Prevalence of *Plasmodium falciparum* haplotypes associated with resistance to sulfadoxine–pyrimethamine and amodiaquine before and after upscaling of seasonal malaria chemoprevention in seven African countries: a genomic surveillance study. *Lancet Infect Dis* 2023; **23:** 361–70.
10 WHO. Management of severe malaria: a practical handbook. Geneva: World Health Organization, 2013.
11 Roper C, Pearce R, Bredenkamp B, et al. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet* 2003; **361:** 1174–81.
12 Goudet J. FSTAT (version 1.2): a computer program to calculate F-statistics. *J Hered* 1995; **86:** 485–86.
13 Oyola SO, Ariani CV, Hamilton WL, et al. Whole genome sequencing of *Plasmodium falciparum* from dried blood spots using selective whole genome amplification. *Malar J* 2016; **15:** 597.
14 Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009; **25:** 1754–60.
15 Ahouidi A, Ali M, Almagro-Garcia J, et al. An open dataset of *Plasmodium falciparum* genome variation in 7,000 worldwide samples. *Wellcome Open Res* 2021; **6:** 42.
16 Jombart T, Ahmed I. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* 2011; **27:** 3070–71.
17 Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010; **26:** 841–42.
18 Alam MT, Vinayak S, Congpuong K, et al. Tracking origins and spread of sulfadoxine–resistant *Plasmodium falciparum* dhps alleles in Thailand. *Antimicrob Agents Chemother* 2011; **55:** 155–64.
19 Beghain J, Langlois A-C, Legrand E, et al. *Plasmodium* copy number variation scan: gene copy numbers evaluation in haploid genomes. *Malar J* 2016; **15:** 206.

20 Lee S, Bahlo M. moimix: an R package for assessing clonality in high-throughput sequencing data. 2016. https://github.com/bahlolab/moimix (accessed Oct 5, 2023).

21 Witkowski B, Duru V, Khim N, et al. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. *Lancet Infect Dis* 2017; **17:** 174–83.

22 Claessens A, Harris LM, Stanojcic S, et al. RecQ helicases in the malaria parasite *Plasmodium falciparum* affect genome stability, gene expression patterns and DNA replication dynamics. *PLoS Genet* 2018; **14:** e1007490.

23 Naidoo I, Roper C. Drug resistance maps to guide intermittent preventive treatment of malaria in African infants. *Parasitology* 2011; **138:** 1469–79.

24 van Eijk AM, Larsen DA, Kayentao K, et al. Effect of *Plasmodium falciparum* sulfadoxine–pyrimethamine resistance on the effectiveness of intermittent preventive therapy for malaria in pregnancy in Africa: a systematic review and meta-analysis. *Lancet Infect Dis* 2019; **19:** 546–56.

25 Gosling RD, Gesase S, Mosha JF, et al. Protective efficacy and safety of three antimalarial regimens for intermittent preventive treatment for malaria in infants: a randomised, double-blind, placebo-controlled trial. *Lancet* 2009; **374:** 1521–32.

26 Desai M, Gutman J, Taylor SM, et al. Impact of sulfadoxine–pyrimethamine resistance on effectiveness of intermittent preventive therapy for malaria in pregnancy at clearing infections and preventing low birth weight. *Clin Infect Dis* 2016; **62:** 323–33.

27 WHO. WHO guidelines for malaria. March 14, 2023. https://apps.who.int/iris/rest/bitstreams/1493946/retrieve (accessed April 10, 2023).

28 Deutsch-Feldman M, Aydemir O, Carrel M, et al. The changing landscape of *Plasmodium falciparum* drug resistance in the Democratic Republic of Congo. *BMC Infect Dis* 2019; **19:** 872.

29 Rosillo SR, Dimbu PR, Cândido ALM, et al. Molecular markers of sulfadoxine–pyrimethamine resistance in samples from children with uncomplicated *Plasmodium falciparum* at three sites in Angola in 2019. *Antimicrob Agents Chemother* 2023; **67:** e0160122.

30 Zhao D, Zhang H, Ji P, et al. Surveillance of antimalarial drug-resistance genes in imported *Plasmodium falciparum* isolates from Nigeria in Henan, China, 2012–2019. *Front Cell Infect Microbiol* 2021; **11:** 644576.

31 Plowe CV. The evolution of drug-resistant malaria. *Trans R Soc Trop Med Hyg* 2009; **103** (suppl 1)**:** S11–14.

32 Cohen O, Guemas E, Menard S, et al. Effect of sulfadoxine–pyrimethamine chemoprophylaxis in pregnant women on selection of the new *P. falciparum dhps* quintuple mutant carrying the I431V mutation. *J Antimicrob Chemother* 2023; **78:** 665–68.

33 Ndu A, Mbachu C, Anitube O, Ezeoke U. Inequities in the use of sulphadoxine-pyrimethamine for malaria prophylaxis during pregnancy in Nigeria. *Malawi Med J* 2020; **32:** 45–51.